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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

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TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

DE1292

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/913392 ✓

INTERNATIONAL APPLICATION NO.

PCT/KR00/00104

INTERNATIONAL FILING DATE

FEBRUARY 11, 2001 2001

PRIORITY DATE CLAIMED

FEBRUARY 11, 1999

TITLE OF INVENTION

AVIAN PLURIPOTENT EMBRYONIC GERM CELL LINE ✓

APPLICANT(S) FOR DO/EO/US

Jae Yong HAN and Tae-Sub PARK ✓

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☒ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☒ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☒ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☒ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

specification including description, claims and abstract
PCT Request
PCT Demand
PCT Written Opinion

09/913392

PCT/KR00/00104

DE1292

24. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☒ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **\$1000.00**
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **\$860.00**
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **\$710.00**
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **\$690.00**
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **\$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =

CALCULATIONS PTO USE ONLY

\$1,000.00Surcharge of **\$130.00** for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492 (e)).☐ 20☐ 30**\$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	26 - 20 =	6	x \$18.00	\$108.00
Independent claims	4 - 3 =	1	x \$80.00	\$80.00

Multiple Dependent Claims (check if applicable).

☐**\$0.00****TOTAL OF ABOVE CALCULATIONS = \$1,188.00**

☒ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.

\$0.00**SUBTOTAL = \$1,188.00**Processing fee of **\$130.00** for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492 (f)).☐ 20☐ 30**\$0.00****TOTAL NATIONAL FEE = \$1,188.00**

Fees for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).

☒**\$40.00****TOTAL FEES ENCLOSED = \$1,228.00**

Amount to be refunded	\$
charged	\$

☐ A check in the amount of _____ to cover the above fees is enclosed.

☒ Please charge my Deposit Account No. **01-1944** in the amount of **\$1,228.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **01-1944**. A duplicate copy of this sheet is enclosed.

☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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RICHARD B. KLAR

NAME

31,385

REGISTRATION NUMBER

August 10, 2001

DATE

09/913392

THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT : Jae Yong HAN, et al.
SERIAL NO. : to be assigned
FILED : herewith
FOR : AVIAN PLURIPOTENT EMBRYONIC GERM CELL
LINE
EXAMINER : Not available GROUP: not available

Commissioner for Patents
Washington, D.C. 20231

Box: Patent Application

PRELIMINARY AMENDMENT

Sir:

Prior to examination, please amend the above-identified patent application as follows:

In the Claims:

Please amend the following claim 13, a clean copy of which is enclosed in the attached appendix as follows:

-- 13 (Amended) The process of claim 1 [or 12], wherein the feeder layer is fibroblast or an equivalent thereof. --

Please add new dependent claim 26 as presented in the attached appendix:

REMARKS

This Amendment is being filed prior to examination.

Amended claim 13 and added dependent claim 26 obviate the need for multiple dependent claim fees.

An early favorable allowance of the claims remaining in the present application is earnestly solicited.

Respectfully submitted,



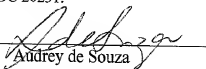
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August 10, 2001

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CERTIFICATE OF MAILING

I hereby certify that this PRELIMINARY AMENDMENT and CONTINUATION APPLICATION W/ATTACHMENTS are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR § 1.10 on August 10, 2001 Mailing Label Number: EL 763941738 US, and is addressed to the Commissioner for Patents, Box Patent Application, Washington, DC 20231.


Audrey de Souza

APPENDIX

Amended Claim:

13 The process of claim 1, wherein the feeder layer is fibroblast or an equivalent thereof.

New Claim:

26. The process of claim 12, wherein the feeder layer is fibroblast or an equivalent thereof.

AVIAN PLURIPOTENT EMBRYONIC GERM CELL LINECROSS REFERENCE TO RELATED APPLICATION

- 5 This application is a continuation patent application of PCT Patent Application No. PCT/KR00/00104, which was filed on February 11, 2000, designating the United States of America.

FIELD OF THE INVENTION

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The present invention relates to a process for preparing an established avian pluripotent embryonic germ cell line and an avian pluripotent embryonic germ cell line.

15 BACKGROUND OF THE INVENTION

- Embryonic stem (ES) cell lines are undifferentiated and pluripotent cells isolated from blastocyst or morula embryos. Although ES cells are expected to be highly useful, e.g., in the study of developmental biology, analysis of the characteristics of totipotent cells, and gene-targeting to produce genetically modified livestock, only mouse ES cells with proven germ-line transmission have been established (Evans, M. J. and Kaufman, M. H., *Nature*, 292, 154-156(1981); Bradley et al., *Nature*, 309, 255-256(1984)) and the use of ES cells in producing livestock has not yet become a reality because of limitations in germ-line transmission in species other than mice (First, N. L. et al., *Reprod. Feril. Dev.*, 6, 553-562(1994); wheeler, M. B., *Reprod. Fertil. Dev.*, 6, 563-568(1994); Giles, J. R. et al., *Mol. Reprod. Dev.*, 36, 130-138(1993); and Doetschman, T. C. et al., *Dev. Biol.*, 127, 224-227(1988)).

- 20 Recently, primordial germ cells (PGCs), which are the progenitors of sperm or egg cells that develop after sexual maturity, have been provided as an alternative source of pluripotent stem cells. The stem cells derived from PGCs are called embryonic germ (EG) cells (Resnick, J. L. et al., *Nature*, 359, 550-551(1992); and Matsui, Y. et al., *Cell*, 70, 841-847(1992)). Mouse PGCs

have been successfully co-cultured on mitotically inactivated STO cells supplemented with three critical growth factors: stem cell factor (SCF), leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF) (Godin, J. R. et al., *Mol. Reprod. Dev.*, 36, 130-138(1991); Dolci, S. et al.,
5 *Nature*, 352, 809-811(1991); Matsui, Y. et al., *Nature*, 353, 750-752(1991); and Resnick, J. L. et al., *Nature*, 359, 550-551(1992)). Resnick et al. reported that mouse PGCs continued to proliferate after subculture and formed colonies of cells that resembled ES cells (Resnick, J. L. et al., *Nature*, 359, 550-551(1992)). Labosky et al. demonstrated that murine EG cell lines had the pluripotency
10 needed for germ-line transmission in vivo (Labosky, P. A. et al., *Development*, 120, 3197-3204(1994)).

As to bovine and porcine EG cells, there have been characterized traits such as morphology, alkaline phosphatase activity, and embryoid body formation (Cherny, R. A. et al., *Reprod. Fertil. Dev.*, 6, 569-575(1994); Shim, H.
15 et al., *Biol. Reprod.*, 57, 1189-1095(1997); Piedrahita, J. A. et al., *J. Reprod. Fertil.*, 52, 245-254(1997)). However, germ-line transmission has not been proven in these species.

Pain et al. reported that avian stem cells with multiple morphogenetic potentialities were derived and maintained in vitro by long-term culture of
20 blastodermal cells. However, the production of pluripotent EG cells derived from PGCs has not previously been reported in any non-mammalian species.

In avian species, PGCs first arise from the epiblast and migrate to the hypoblast of the area pellucida (the germinal crescent) at stage 4, approximately 18-19 hours after incubation (Swift, C. H., *Am. J. Anat.*, 15,
25 483-516(1914); Hamburger, V. and Hamilton, H. L., *J. Morphol.*, 88, 49-92(1951); and Eyal-Giladi, H. and Kochav, S., *Dev. Biol.*, 49, 321-337(1976)). PGCs move from the germinal crescent into the blood stream at stage 10-12 (Ando, Y. and Fujimoto, T., *Dev. Growth Differ.*, 25, 345-32(1983); and Ukeshima, A. et al., *J. Electron. Microsc.*, 40, 124-128
30 (1991)) and circulate until stage 17 (2.5 days of incubation) when they reach the region of the germinal ridges, in which they finally concentrate and

colonize (Nieuwkoop, P. D. and Sutasurya, L. A., In Primordial Germ Cells in the Chordates, 113-127(1979)). This migration pathway and the subsequent developmental processes differ dramatically from the comparable processes
5 in mammalian species.

Allioli et al. reported that chicken PGCs isolated from gonads could proliferate for several days under an in vitro culture condition (Allioli, N. et al., Dev. Biol., 165, 30-37(1994)). Chang et al. cultured chicken PGCs from gonads
10 on stroma cells of the germinal ridge for 5 days (Chang, I. et al., Cell Biol. Int., 19, 569-676(1995)). These cultured gonadal PGCs had the ability to migrate to the germinal ridge when re-injected into recipient embryos. Recently, Chang et al. produced germ-line chimeric chickens by
15 injection of gonadal PGCs which had been cultured in vitro for 5 days (Chang, I. et al., Cell Biol. Int., 21, 495-499(1997)).

Further, PCT publication No. WO 99/06534 discloses a method for establishing EG cells by culturing PGCs isolated
20 from the blood of an embryo at stage 13 to 14 in a medium supplemented with LIF, bFGF, SCF and IGF-I without using a feeder layer. However, the amount of blood PGCs obtainable from an embryo, i.e., about 100 cells/embryo, is much less than that of gPGCs, i.e., more than 1000 cells/embryo, and
25 it is difficult to isolate the blood PGCs from the embryonic blood. Further, although the PGCs were cultured without using a feeder layer, they attached to the wall of the culture vessel and morphologically changed only after 3 or 4 passages. Accordingly, it is presumed that the EG cells
30 established therefrom would be already differentiated. Moreover, the EG cells were examined only on their alkaline phosphatase activity for the demonstration of the pluripotency thereof without examination on other properties and, accordingly, it is not certain whether the EG cells are
35 indeed established. In addition, the reference did not teach or anticipate whether the EG cells have the in vitro and in vivo differentiating abilities, which are characteristics of

an established EG cells, and whether they proliferate continuously after several passages.

Thus, there has continued to exist a need to develop an EG cell line transmitting foreign gene through multiple generations, thereby enabling the production of a transgenic livestock using foreign gene transfection and gene targeting.

SUMMARY OF THE INVENTION

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Accordingly, it is an object of the present invention to provide a process for establishing an avian pluripotent embryonic germ (EG) cell line.

Another object of the present invention is to provide an avian pluripotent embryonic germ cell line.

In accordance with one aspect of the present invention, there is provided a process for preparing an established avian embryonic germ cell line comprising the steps of:

(a) culturing primordial germ cells (PGCs) isolated from an avian embryonic gonad in a medium supplemented with a cell growth factor and a differentiation inhibitory factor to obtain EG cell colonies;

(b) culturing the EG cells in the same medium as in step (a) by employing a feeder layer until the EG cells are colonized; and

(c) recovering and subculturing the EG cells in the same medium as in step (a) to establish the EG cell line.

In accordance with another aspect of the present invention, there is provided a chicken embryonic germ cell line having characteristics substantially identical to that deposited under the accession number of KCLRF-BP-00026.

BRIEF DESCRIPTION OF THE DRAWING

35

The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with

the accompanying drawing, in which:

Figs. 1a and 1b show the morphological characteristics of chicken EG cell colonies after 3 passages on chicken embryonic fibroblast (Fig. 1a: scale, 50 μ m; and Fig. 1b: scale, 25 μ m);

Fig. 2 presents the PAS reactivity in EG cell colonies formed after 4 passages;

Fig. 3 presents the anti-SSEA-1 antibody screening results in EG cell colonies formed after 4 passages;

Fig. 4 presents the proliferation assay result in EG cell colonies stained after 8 passages;

Fig. 5a displays the embryoid bodies formed from a chicken EG cells in suspension culture after 8 days; and Figs. 5b to 5d, the results of immunohistochemical analyses of the embryoid bodies using antibodies against actin, α -1-fetoprotein and S100, respectively;

Fig. 6a exhibits typical Korean Ogol chickens having black feather; and Fig. 6b shows a somatic chimera chicken having white patches around the neck and on the breast; and

Fig. 7 depicts the result of PCR analysis for somatic and germline chimerisms.

DETAILED DESCRIPTION OF THE INVENTION

The process of the present invention for establishing an avian embryonic germ (EG) cell line begins with the isolation of avian primordial germ cells (PGCs). The avian may be turkey, chicken, quail, pheasant or duck. The PGCs may be isolated from the embryonic gonad of an early-stage avian embryo, at stage 14 to 36 (50 hours to 10 days after incubation), preferably, stage 24 to 30 (4 to 6.5 days after incubation). For instance, the embryonic gonad of White Leghorn at stage 28 is isolated and then the gonad tissue is dissociated in trypsin-EDTA to obtain a suspension containing gonadal primordial germ cells (gPGC). However, the developmental stage of the embryo is not limitative unless it impairs the purpose of the present invention, and

may vary according to the avian species or the kind of organ, tissue and membrane from which the PGCs are separated.

The isolated PGCs are cultured at a temperature ranging
5 from 37 °C to 42 °C under an atmosphere containing 5% CO₂ in
a medium containing cell growth factors and a
differentiation inhibitory factor until the colonization of
EG cells. The medium is preferably DMEM (Dulbecco's
Modified Eagle's Medium; Gibco BRL cat#, 10313-021) or a
10 functional equivalent thereof.

Exemplary cell growth factors useful in the present
invention include stem cell factor (SCF), basic fibroblast
growth factor (bFGF), interleukin-11 (IL-11), insulin-like
growth factor-I (IGF-I) and a mixture thereof.
15 Representative differentiation inhibitory factor is leukemia
inhibitory factor (LIF). The culture medium may further
contain mammalian serum, avian serum or a supplementary
ingredient selected from the group consisting of sodium
pyruvate, glutamine, β-mercaptoethanol and a mixture
20 thereof.

The culture medium is preferably DMEM supplemented with
0.1 to 30% fetal bovine serum (FBS), 0.02 to 20 % chicken
serum, 0.01 to 100 mM sodium pyruvate, 0.02 to 200 mM
glutamine, 0.55 to 5500 μM β-mercaptoethanol, 0.05 to 500
25 ng/ml of SCF, 0.1 to 1000 units/ml of LIF, 0.1 to 1000 ng/ml
of bFGF, 0.0004 to 4 ng/ml of IL-11 and 0.1 to 1000 ng/ml of
IGF-1.

The EG cell colonies formed on the medium is separated
into individual EG cell, e.g., by repeated pipetting, and
30 the EG cells are recovered, suspended in a medium, e.g., the
medium described above, and then cultured at 37 to 42 °C for
7 to 10 days on a feeder layer until the colonization of
cells exhibiting the morphological characteristics of an EG
cell line. As the feeder layer, avian embryonic fibroblast
35 or an equivalent thereof may be employed, while mitotically
active avian embryonic fibroblast (CEF) or avian fibroblast
is most preferred.

The resulting EG cells are passaged at an interval of 7 to 10 days in the same medium as above to establish an embryonic germ cell line. The embryonic germ cell line thus established may be maintained for a period of over 4 months by repeated subculture.

The present invention also provides a chicken EG cell line produced by using the inventive process.

The morphology of the chicken EG cell line colonies is multi-layered and the colonies are well-delineated. Each chicken EG cell is composed of a large nucleus and a relatively small amount of cytoplasm, similar to the separated morphologies of murine and porcine ES cells and EG cells (Wobus, A. M. et al., Exp. Cell. Res., 152, 212-2199(1984); Matsui, Y. et al., Cell, 70, 841-847(1992); Resnick, J. L. et al., Nature, 352, 550-551(1992); Shim, H. et al., Biol Reprod, 57, 1089-1095(1997); and, Piedrahita, J. A. et al., Biol. Reprod., 58, 1321-1329(1998)). However, the morphology of the chicken EG cells is slightly different from that of mouse ES or EG cells in that almost all of the colonies are uniformly round and that the prominent dark nucleoli observed in other species are not clearly discernible. Avian species are different from mammalian species in terms of physiology, development and differentiation of germ cells and, thus, the chicken EG cells are different from mouse EG cells in their shape, their adhesive properties, and other characteristics.

The chicken EG cells maintain the characteristics of gonadal PGCs and undifferentiated stem cells. The chicken EG cells express SSEA-1 antigen and, in an in vitro suspension culture, successfully develop into embryoid bodies which differentiate into a variety of cell types. Further, the chicken EG cells are confirmed to proliferate and differentiate into various tissues including the gonad during embryo development in an experiment using chickens and, thus, they are pluripotent in vivo.

One of the chicken embryonic germ cell lines established in the present invention was deposited on

September 22, 1999 with the Korean Cell Line Research Foundation(KCLRF) (Address: Cancer Research Institute, Seoul National University College of Medicine, #28, Yongon-dong, Chongno-gu, Seoul, 110-744, Republic of Korea) under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganism for the Purpose of Patent Procedure under the accession number of KCLRF-BP-00026.

A somatic or germline chimera can be produced by microinjection of EG cells into an egg, preferably, into the germinal cavity or blood vessel thereof. More preferably, the EG cell may be microinjected into the germinal cavity of an egg at stage X or into the blood vessel of an egg at stage ranging from 13 to 17.

A desired foreign gene can be transfected and introduced in the inventive chicken EG cells by electroporation or liposome, and stably-transfected chicken EG cells can be selected by passaging them in a medium containing an antibiotic.

Thus, the inventive chicken EG cell lines are useful for the production of transgenic chickens and for studies of germ cell differentiation and genomic imprinting.

The following Examples are intended to further illustrate the present invention without limiting its scope.

Further, percentages given below for solid in solid mixture, liquid in liquid, and solid in liquid are on a wt/wt, vol/vol and wt/vol basis, respectively, unless specifically indicated otherwise.

Example 1 : Isolation of PGCs and establishment of culture condition for preparing a chicken EG cell line

(Step 1) Isolation of PGCs

A fertilized egg of White Leghorn obtained from the College of Agriculture and Life Sciences, Seoul National University was incubated for 5.5 days (until stage 28) at 37.5 °C and a relative humidity of 60-70%. The embryo was

extracted from the fertilized egg at stage 28 and washed in a 100 mm petri dish with magnesium-free phosphate buffered saline (PBS) to remove the yolk and blood. The embryo was transferred to a petri dish coated with a black wax and the
5 embryonic gonads were isolated therefrom with forceps. The gonad tissue was separated into individual gonadal primordial germ cells (gPGCs) by treating with 0.25% trypsin-0.05% EDTA. Added thereto was DMEM (Dulbecco's Modified Eagle's Medium, Gibco BRL, USA) containing 10% FBS
10 (fetal bovine serum, Gibco, USA) to inactivate trypsin-EDTA, and gPGCs were harvested by centrifugation.

(Step 2) Establishment of culture condition

The EG (embryonic germ) cell culture medium (i.e.,
15 DMEM) was supplemented with one or more of growth factors selected from stem cell factor (SCF), leukemia inhibitory factor (LIF), and basic fibroblast growth factor (bFGF). SCF, LIF, and bFGF have been reported to be important growth factors for the survival and proliferation of PGCs in the
20 mouse (Resnick, J. L. et al., Nature, 359, 550-551(1992); and Donovan, P. J., Curr. Top. Dev. Biol., 29, 189-225(1994)). In addition to these three factors, growth-related factors such as interleukin-11 (IL-11) and insulin-like growth factor-I (IGF-I) were added to the
25 medium. The gPGCs obtained in Step 1 were suspended in the culture medium supplemented with various combinations of the above factors and then incubated at 37 °C under an atmosphere containing 5% CO₂ until the colonization of the EG cells.

30 Tests showed that colonization of EG cells does not occur in the absence of IL-11 and IGF-I. Therefore, IL-11 and IGF-I are essential for the survival and proliferation of chicken EG cells.

35 Example 2 : Culture of chicken EG cells

The EG cells obtained in Example 1 were seeded in a 24-

well culture plate containing EG cell culture media consisting of DMEM (Gibco, USA) supplemented with 10% FBS, 2% chicken serum (Gibco, USA), 1mM sodium pyruvate, 2mM L-glutamine, 5.5×10^{-5} M β -mercaptoethanol, 100 μ g/ml of streptomycin, 100 units/ml of penicillin, 5 ng/ml of human stem cell factor (hSCF; Sigma, USA), 10 units/ml of murine leukemia inhibitory factor (mLIF; Sigma, USA), 10 ng/ml of bovine basic fibroblast growth factor (bFGF; Sigma, USA), 0.04 ng/ml of human interleukin-11 (h-IL-11; Sigma, USA) and 10 ng/ml of human insulin-like growth factor-I (IGF-I; Sigma, USA) and incubated in an incubator for 7-10 days at 37°C under an atmosphere of 5% CO₂ to produce EG cell colonies deposited on a layer of germinal ridge stroma cells (GRSC). The colonies of chicken EG cells were separated from the GRSC layer by gentle pipetting and harvested by centrifuge at 200 X g for 5 minutes. The harvested EG cells were suspended in DMEM and divided into a fresh 24-well plate together with chicken embryonic fibroblasts (CEFs) which were not mitotically inactivated. The EG cell colonies were passaged at an interval of 7 to 10 days under the condition as above. These colonies were maintained for up to 10 passages and proliferated over a period of 4 months in repeated subculture.

Figs. 1a and 1b show chicken EG cell colonies after 3 passages on chicken embryonic fibroblast (CEF) cells (Fig. 1a: scale, 50 μ m; and Fig. 1b: scale, 25 μ m). The morphology of the chicken EG cells was slightly different from that of mouse ES or EG cells. Almost all of the chicken EG cell colonies were uniformly round and were not tightly bound to the CEF feeder layer. In contrast to mouse ES or EG cells, chicken EG cells did not pack strongly together and; it was not difficult to discern the individual component cells. The morphology of the colonies was multi-layered and the boundaries thereof was well-delineated. The chicken EG cell was composed of a large nucleus and a relatively small amount of cytoplasm, while its nucleoli was not prominent.

Example 3: Characterization of the EG cell

To determine whether the pluripotency of the EG cell possessed characteristic features of a pluripotent cell, the presence of glycogens and SSEA-1 epitope as well as its alkaline phosphatase activity and abilities to proliferate and differentiate in vitro were examined.

(1) Periodic Acid-Shiff's (PAS) staining

10

The EG cell colonies after 4 passages obtained in Example 2 were fixed to a plate in a 1 % glutaraldehyde solution for 5 min. and washed twice with an equal volume of phosphate buffered saline (PBS). The EG cell colonies were immersed in Periodic Acid Solution (Sigma, USA) at room temperature for 5 min. and then washed with an equal volume of PBS. The EG cell colonies were then immersed in Shiff's Solution (Sigma, USA) at room temperature for 15 min. and then washed twice with an equal volume of PBS. The resulting EG cell colonies were observed under an inverted microscope. Chicken PGCs can be easily identified by the PAS reaction which stains glycogens in the cytoplasm (Meyer, D. B., Dev. Biol., 10, 154-190 (1964)). As can be seen in Fig. 2, the EG cells after 4 passages can be stained by PAS, although the staining is relatively weak.

(2) Anti-SSEA-1 antibody screening

The SSEA-1 epitope is characteristic of undifferentiated murine ES cells and has been as a criterion for distinguishing pluripotent stem cells (Solter, D. et al., Proc. Natl. Acad. Sci., 75, 5565-5596 (1978)). To determine whether the pluripotency of the EG cell possessed characteristic features of a pluripotent cell, the presence of SSEA-1 epitope were examined.

The EG cell colonies after 4 passages obtained in Example 2 were fixed to a plate in 1 % glutaraldehyde

solution for 5 min. and washed twice with an equal volume of PBS. An ascites fluid of anti-SSEA-1 monoclonal antibody (MC-480; Solter, D. et al., Proc. Natl. Acad. Sci., 75, 5565-5596 (1978)) was purchased from the Development Studies Hybridoma Bank, Iowa University U.S.A., diluted 1,000 fold with PBS, and added to the EG cell colonies. The resulting EG cell colonies were reacted with avidin/biotin-conjugated alkaline phosphatase (Vector Lab., USA) and then with BCIP/NBT alkaline phosphatase substrate (BCIP/NBT alkaline phosphatase substrate kit IV, Vector Lab., USA). The reaction was stopped by adding 10 mM EDTA (pH 8.0) thereto.

As can be seen in Fig. 3, all EG cells after 4 passages are positive toward SSEA-1 staining showing that the SSEA-1 epitope is expressed by the chicken EG cells.

(3) Proliferation assay

The EG cell colonies after 8 passages obtained in Example 2 were kept in solution containing bromodeoxyuridine (BrdU, a thymidine analog), at 37 °C for 1 hour and washed twice with an equal volume of PBS. The resulting EG cell colonies were stained by cell proliferation assay kit (Amersham, UK) and then counterstained with PAS by repeating the procedure of (1). EG cell colonies containing incorporated BrdU were detected using any anti-BrdU monoclonal antibody and a peroxidase/DAB system (Amersham, UK).

Fig. 4 presents the proliferation assay result of EG cell colonies stained after 8 passages. As can be seen from Fig. 4, the EG cells after 8 passages are in continuous proliferation.

(4) Alkaline phosphatase activity assay

The alkaline phosphatase activity of the EG cell colonies after 4 passages obtained in Example 2 was measured using alkaline phosphatase substrate kit IV (Vector Lab.,

USA).

The result shows that the EG cells have scarcely alkaline phosphatase activity and did not regain the activity during subculture.

5 According to Swartz, W. J., Anat. Rec., 202, 379-385 (1982), alkaline phosphatase activity of avian PGCs is observably as early as 2 days of incubation but not after the entry of PGCs into genital ridge, which would suggest that the alkaline phosphatase activity of gonadal PGCs as
10 well as EG cells passaged therefrom would indeed be very weak.

(5) In vitro differentiation and immunohistochemical analysis

15 To examine whether embryoid bodies could formed from the chicken EG cells, the EG cell colonies after 4 passages obtained in Example 2 were gently agitated and centrifuged to obtain individually separated EG cells. The EG cells
20 were suspended in EG cell culture media free from mLIF and then placed in a non-adhesive bacteriological petri dish. The media was changed every other day for 8 days and the morphology of the cells was monitored daily.

Fig. 5a displays the embryoid bodies formed from
25 chicken EG cells in suspension after 8 days.

The embryoid bodies thus formed were collected and then distributed into a 96-well plate further to attach thereto and differentiate. The resulting cells were subjected to immunohistochemical analysis using antibodies for muscle-specific actin (Dako, USA), endoderm-specific α -1-
30 fectoprotein (Dako, USA) and ectoderm-specific S100 (Dako, USA), together with an DAKO LSAB kit and avidin/biotin-conjugated peroxidase system (Dako, USA).

Figs. 5b to 5d illustrate the results of
35 immunohistochemical analyses of the embryoid bodies using antibodies against actin, α -1-lectoprotein and S100, respectively. As can be seen from Figs. 5b to 5d, the EG

cells are capable of differentiating in vitro into a variety of cell types, e.g., endoderm, mesoderm and ectoderm lineage.

5 Example 4: Production of Chimeric Chicken

Korean Ogol chicken embryos at stage X or 13 to 17 (Eyal-Giladi, H. et al., Dev Biol, 49, 321-337(1976)) were used as recipients. The lateral part or pointed end of each
10 Korean Ogol chicken egg was punctured to provide a small window and then the shell membrane was removed.

The EG cells after 3 or 4 passages obtained in Example 2 were suspended in EG cell culture media at a concentration of 10^3 cells/ μ l and then 2 μ l of the suspension was injected
15 into the germinal cavity or blood vessel of the egg using a micropipette. Eggs injected with CEF were used as a control. The window of each egg was sealed twice with paraffin film, and then, the eggs were laid down with the pointed end at the bottom until hatching. The hatched
20 chicks were allowed to grow for 3 months to obtain somatic chimera chickens.

Of 45 eggs with manipulated embryos, 8 eggs were hatched and, among the eight, 3 chicks (37.5 %) were externally chimeric. Among chimeric chicks, two white
25 patched chicks were hatched from the embryos injected with the EG cells of 3 passages and one was from the embryo injected with the EG cells of 4 passages. The extent of white patching varied significantly among the 3 chicks. No white feather was observed with the control chick.

Fig. 6a exhibit, a pair of typical Korean Ogol chicken having black feathers; and Fig. 6b, the somatic chimera chicken having white patches around the neck and on the breast. The feather color of a White Leghorn is white due to the dominant pigmentation inhibitory gene(I/I), and a
35 Korean Ogol chicken, black due to the recessive pigment gene(i/i). Accordingly, the above result suggests that the EG cells of the White Leghorn are capable of differentiating

in vivo in the recipient Korean Ogol chicken embryo and, therefore, the EG cells are pluripotent in vivo. To verify the somatic chimerism of the chicken, genomic DNA samples were obtained by phenol extraction from the muscle, heart, liver and gonad of 5 chicks which died during hatching and then subjected to PCR analysis using White Leghorn-specific SCAR (sequence characterized amplified region) primers having nucleotide sequences of SEQ ID NO: 1 (forward primer) and SEQ ID NO: 2 (reverse primer).

10 PCR reactions were carried out at a 25 μ l scale using 50 to 100 ng of genomic DNA, 0.2 mM of each dNTP, 10 mM KCl, 1.5 mM $MgCl_2$, 0.4 pmol of forward primer, 0.4 pmol of reverse primer and 1 unit of Taq polymerase. The PCR program consisted of 1 minute of denaturation at 94 $^{\circ}C$, 1
15 minute of annealing at 60 $^{\circ}C$ and 2 minutes of extension at 72 $^{\circ}C$ for 45 cycles in a DNA thermocycler (Perkin Elmer Cetus). The White Leghorn specific DNA fragment produced was approximately 3 kb. Fig. 7 depicts the results of PCR analysis for somatic chimerism: Lanes 1, 5, 9, 13 and 17,
20 PCR products using the liver DNA; Lanes 2, 6, 10, 14 and 18, the muscle DNA; Lanes 3, 7, 11, 15 and 19, the heart DNA; Lanes 4, 8, 12, 16 and 20, the gonad DNA; Lane 21, Korean Ogol chicken genomic DNA; Lane 22, White Leghorn genomic DNA; and Lane 23, no template. As can be seen from Fig. 7,
25 the somatic chimerisms were different between individuals: one of the 5 chicks showed somatic chimerism in all tissue samples (liver, heart, muscle, and gonads). The injected EG cells contributed to the gonads of two hatched chicks and the heart in all of the chicks; two chicks showed the
30 somatic chimerism only in the heart. These results indicate that chicken EG cells can differentiate and contribute in vivo to various tissues including the gonads.

Example 5: Transfection of Foreign Gene into PGCs or EG
35 cells and Selection Thereof

A reporter gene (GFP or Lac Z) was transfected into

PGCs or EG cells using electroporation and liposome, respectively. Transfection efficiency of the gene was about 80% in case of using electroporation and 30% in case of using liposome. Transfected PGCs or EG cells were passaged
5 in a DMEM medium containing 350 μ g/ml of neomycin to select the stably transfected PGCs or EG cells.

While the invention has been described with respect to the above specific embodiments, it should be recognized that
10 various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEPTION IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1

To: Jae Yong Han

Dongbo APT 101-513, Yongin 3 cha, Suji, Yongin-City,
Kyonggi-do 449-840, KOREA

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the
DEPOSITOR : CEG (chicken embryonic
germ cell line)

Accession number given by the
INTERNATIONAL DEPOSITARY
AUTHORITY:
KCLRF-BP-00026

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by :

☒ A scientific description

☒ A proposed taxonomic designation

(Mark with a cross where applicable)

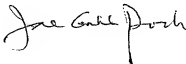
III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I
above, which was received by it on September, 22, 1999

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name : Director
Korean Cell Line Research
Foundation
Address : Cancer Research Institute
Seoul National University
College of Medicine
28 Yongon-dong, Chongno-Gu
Seoul, 110-744, Korea

Signature(s) :



Date : 1999. 10. 25

What is claimed is:

1. A process for preparing an established avian embryonic germ (EG) cell line comprising the steps of:

- 5 (a) culturing primordial germ cells (PGCs) isolated from an avian embryonic gonad in a medium supplemented with a cell growth factor and a differentiation inhibitory factor to obtain EG cell colonies;
- (b) culturing the EG cells in the same medium as in
10 step (a) by employing a feeder layer until the EG cells are colonized; and
- (c) recovering and subculturing the EG cells in the same medium as in step (a) to establish the EG cell line.

15 2. The process of claim 1, wherein the avian embryonic gonad is at a stage ranging from 14 to 36.

3. The process of claim 2, wherein the avian embryonic gonad is at a stage ranging from 24 to 30.

20 4. The process of claim 1, wherein the avian species is turkey, chicken, quail, pheasant or duck.

5. The process of claim 1, wherein a layer of
25 germinal ridge stroma cells (GRSCs) is employed as a feeder layer when culturing primordial germ cells in step (a).

6. The process of claim 1, wherein the growth factor
30 is selected from the group consisting of stem cell factor (SCF), basic fibroblast growth factor (bFGF), interleukin-11 (IL-11), insulin-like growth factor-I (IGF-I) and a mixture thereof.

7. The process of claim 1, wherein the medium is
35 supplemented with a growth factor selected from the group consisting of 0.05 to 500 ng/ml of SCF, 0.1 to 1000 ng/ml of bFGF, 0.0004 to 4 ng/ml of IL-11, 0.1 to 1000 ng/ml of IGF-I

and a mixture thereof.

8. The process of claim 1, wherein the differentiation inhibitory factor is leukemia inhibitory factor (LIF).

9. The process of claim 8, wherein the amount of LIF is 0.1 to 1000 units/ml.

10. The process of claim 1, wherein the medium further comprises mammalian or avian serum.

11. The process of claim 1, wherein the medium further comprises a supplementary ingredient selected from the group consisting of sodium pyruvate, glutamine, β -mercaptoethanol and a mixture thereof.

12. The process of claim 1, wherein the feeder layer is mitotically active.

13. The process of claim 1 or 12, wherein the feeder layer is fibroblast or an equivalent thereof.

14. The process of claim 13, wherein the fibroblast is avian fibroblast or avian embryonic fibroblast.

15. The process of claim 14, wherein the avian species is chicken.

16. An avian embryonic germ (EG) cell line prepared in accordance with the process of claim 1.

17. The avian EG cell line of claim 16, which can be maintained by repeated subculture.

18. The avian EG cell line of claim 16, which expresses SSEA-1 antigen, forms an embryoid body, and

- 20 -

differentiates and contributes to various tissues.

19. The avian EG cell line of claim 16, which is a chicken embryonic germ cell line having characteristics substantially identical to that deposited under the accession number of KCLRF-BP-00026.

20. A process for preparing a somatic or germline chimera comprising injecting the avian EG cell of claim 16 into an egg.

21. The process of claim 20, wherein the EG cell is injected into a germinal cavity or blood vessel of the egg.

22. The process of claim 21, wherein the EG cell is injected into the germinal cavity of the egg at a stage X.

23. The process of claim 21, wherein the EG cell is injected into the blood vessel of the egg at a stage ranging from 13 to 17.

24. A process for transfecting a foreign gene into EG cells characterized by using electroporation or liposome.

25. A process for selecting stably transfected EG cells comprising passaging EG cells transfected by a foreign gene in a medium containing an antibiotic.

FIG. 1a

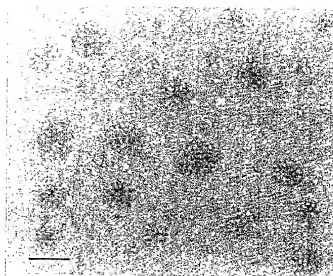


FIG. 1b

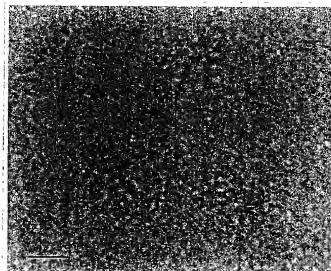


FIG. 2

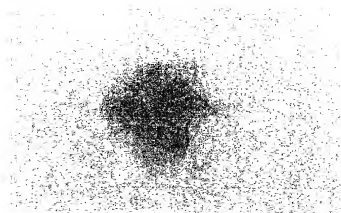


FIG. 3

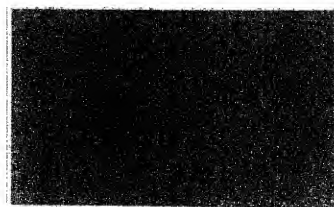


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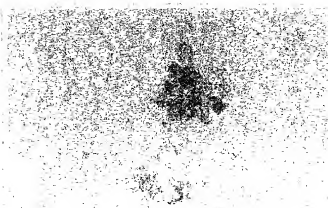


FIG. 5a

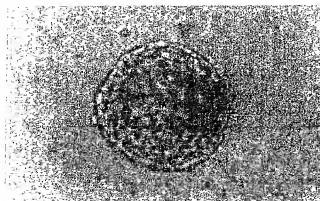


FIG. 5b



FIG. 5c

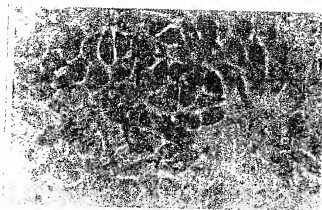


FIG. 5d

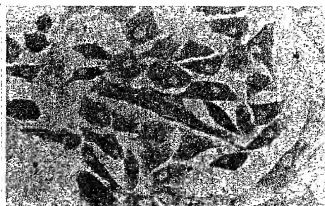


FIG. 6a

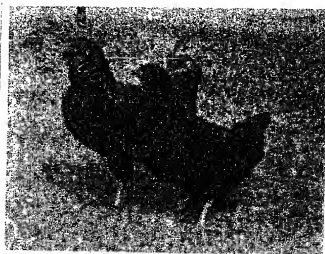


FIG. 6b

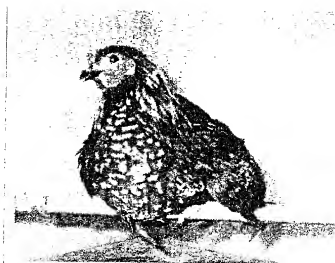
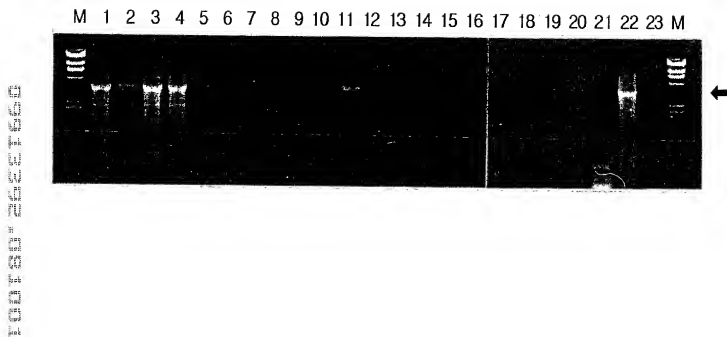


FIG. 7



ANDERSON KILL & OLICK, P.C.
DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

This is a continuation application of International Application No. PCT/KR00/00104, filed on February 11, 2000 and designating the United States of America.

My residence, post office address and citizenship are as stated below my name,

I believe I am the original, first and sole inventor (if only one name is listed at 201 below), or an original, first and joint inventor (if plural names are listed at 201 et seq. below), of the subject matter which is claimed and for which a patent is sought on the invention entitled AVIAN PLURIPOTENT EMBRYONIC GERM CELL LINE

the specification of which:

☒ is attached hereto

☐ was filed on _____ as Application Serial No. _____ (for declaration not accompanying application) with amendment(s) filed _____ (if applicable)

☐ was filed as PCT International application Serial No. _____ on _____ and was amended under PCT Article 19 on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119/§172 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 U.S.C. 119/172
1999-4860 ✓	Republic of Korea ✓	11, 02, 1999 ✓	YES <input checked="" type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION NUMBER	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED
PCT/KR00/00104	11, 02, 2000 ✓		X	

POWER OF ATTORNEY: As a named inventor, I hereby appoint Michael N. Meller (Reg. No. 20,779), Eugene Lieberstein (Reg. No. 24,645), Richard B. Klar (Reg. No. 31,385) and Jack Matalon (Reg. No. 22,441) whose address is **Anderson Kill & Olick, P.C.**, 1251 Avenue of the Americas, New York, New York 10020-1182, as my attorneys, to prosecute this application, and to transact all business in the Patent and Trademark Office connected therewith

Send Correspondence To: David A. Einhorn, Esq.

Direct Telephone Number

Anderson Kill & Olick, P.C.

(212) 278-1000

1251 Avenue of the Americas

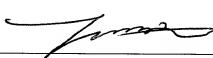

New York, NY 10020

1-00 2 0 1	FULL NAME OF INVENTOR	Last Name	First Name	Middle Name
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	POST OFFICE ADDRESS	Street	City	State or Country
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	RESIDENCE & CITIZENSHIP	City	State of Foreign Country	Country of Citizenship
	POST OFFICE ADDRESS	Street	City	State or Country
				Zip Code

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signature of Inventor 201	Signature of Inventor 202
	
Date	Date
<u>July 26, 2001</u>	<u>July, 26, 2001</u>

09/913392

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PARK, TAE SUB

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PARK, TAE SUB
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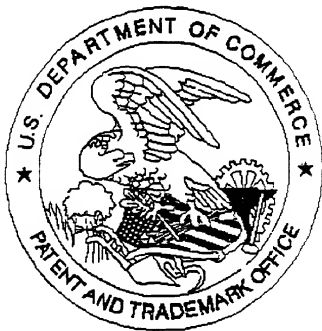
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